PRECURSOR - PRODUCT RELATIONSHIPS IN THYROIDAL IODOCOMPOUNDS

John F. B. Haney

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PRECURSOR-PRODUCT RELATIONSHIPS IN THYROIDAL IODOCOMPOUNDS

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This work is dedicated

to Professor Serge Lissitzky, whose energy, insight and humanity made the period of laboratory investigation so deeply rewarding, and whose hospitality made a year so quickly passed and so fondly remembered,

to all our good friends of his Laboratoire de Biochimie Medicale who gave so much in warmth and camaraderie,

and to those on this side of the Atlantic who helped in ways great and small to make our fellowship year a reality and a success.

For the use of his laboratory, for the gift of his time and materials, and for his guidance in experiment and in the preparation of this paper, the author wishes to thank Dr. Philip K. Bondy.

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CONTENTS

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INTRODUCTION

It is generally accepted that serum inorganic iodide concentrated by the normal mammalian thyroid gland is rapidly and sequentially organified in thyroglobulin into first MIT[2] and then DIT. MIT or DIT is then 'coupled' with DIT contained in thyroglobulin to produce, respectively, T_3 or T_4. These are subsequently released by thyroidal proteases from storage thyroglobulin. Some of the evidence that has helped to form this consensus will be briefly reviewed.

In 1927 Harington and Barger (1) first suggested that thyroxine might arise from the coupling of two molecules of DIT with the elimination of an alanine side chain. Direct experimental support for this suggestion has been considerable (2). A theoretical model (3) for this oxidative conversion and experimental proof (4) that an oxidizing milieu was necessary for the in vitro reaction followed.

Several hypotheses regarding mechanism of formation attended the 1952 discovery (5,6) of T_3 in thyroid tissue. Proponents (7) of the hypothesis that T_3 was formed by intrathyroidal deiodination of T_4 seemed to be disproved by the findings of Roche et al. (8) that thyroidal deiodinase could use only iodotyrosines and not thyroxine as a substrate. However, Pitt-Rivers and Tata later

asserted (9) that when thyroxine was separated from serum proteins it could be deiodinated by pig thyroid deiodinase.

In another approach to the origin of T_3, Plaskett (10,11) determined the distribution of I^{131} on the two rings in T_4 and T_3. From studies of rabbit thyroid tissue hydrolysed 24 hours after the injection of I^{131} he found that the activity was randomly distributed in each ring of the two compounds. He felt that his data were incompatible with the formation of T_3 from MIT and DIT as the labeling in the two rings would have been different unless there were equal activity in MIT and DIT at 24 hours, a circumstance he thought unlikely. (Data are not yet available on whether normal rabbit thyroid has reached isotopic equilibrium 24 hours after I^{131} injection.) He concluded, therefore, that random labeling supported the hypothesis that T_3 was produced by the deiodination of T_4, but that a confident statement could not be made until specific activity data were determined for MIT and DIT.

Precursor-product relationships among these latter compounds are as yet unclear. Michel (12), and later Pitt-Rivers and Tata (13), agree that injected I^{131} appears first in MIT and that as radioactivity in that compound declines the amount in DIT increases, and hence 'MIT is the precursor of DIT.'

Taurog, Tong and Chaikoff (14) investigating another phenomenon--the effect of hypophysectomy on I^{131} incorporation--showed that in thyroid hydrolysates from their normal control rats $MI^{131}T$ activity was maximal and then began to fall off before radioactivity in DIT had reached its maximum and plateaued at that level. This and similar studies are cited as evidence that DIT arises from MIT, as the continuous formation of $DI^{131}T$ would lead to decreasing amounts of MI^{131}. However, in contradiction to these findings Bois and Larsson (15) reported that the DIT/MIT ratio in rat thyroid hydrolysates was constant during a period from 30 minutes to one week after the injection of I^{131}. They concluded, notwithstanding, that DIT was derived from the iodination of MIT, feeling that if there were any other precursors the proportion of $MI^{131}T$ would have decreased with time. Although other workers have reached similar conclusions, the experimental findings of Bois and Larsson have not been substantiated.

Pitt-Rivers (16) studied I^{131} incorporation into MIT, DIT, T_3 and T_4 released by enzymatic hydrolysis from rat thyroid glands, and computed the relative specific activities (RSA) of these compounds. She concluded that MIT and DIT satisfy the

criteria (17) for a precursor-product relationship. The conclusion was also drawn that both T_4 and T_3 are formed by a coupling mechanism from the iodotyrosines, although data are presented that tend to show that the maximum RSA of T_3 occurs before or at the same time as the maximum for MIT, and that the RSA curves of DIT and T_4 likewise are similar in shape and time course.

Pitt-Rivers and Cavalieri (18) soon after indicated that the RSA of free MIT (dialysable at 0^oC) exceeded that of bound ^{3} MIT and the 'second pool of inorganic iodide' up to 6 hours after the injection of a single dose of I^{131}. They found the specific activity of free DIT to be lower than that of free or bound MIT.

If true, these latter findings raise the interesting possibility that he explanation for the simultaneous maxima in the relative specific activities of bound MIT and T_3 and bound DIT and T_4 lies in the participation of the free iodotyrosines or related iodocompounds as well as bound MIT and DIT in hormone biosynthesis.

In the experiments described here it was attempted to clarify the role played by the free iodocompounds reported by Pitt-Rivers and Cavalieri (18), Lissitzky et al. (19), and

Lissitzky and Haney (20) in the biosynthesis of the thyroid hormones by determining their relative specific activities. The free iodocompounds under investigation include, in addition to MIT and DIT (18,19,20) various iodopeptides that have been partially characterized in earlier communications from Lissitzky's group (19,20). Other free iodocompounds, including MIHPPA and DIHPPA, which have been described (21) in thyroidal tissue were not investigated in the present work[4].

MATERIAL AND METHODS

White rats of the Wistar strain were fed on a diet of standard animal biscuit and bread with no special precaution to obtain a low-iodine diet. Males weighing 100-140 grams were injected intraperitoneally with carrier-free NaI^{131} (Commissariat a l'Energie Atomique, Saclay, Seine et Oise, France), killed by a blow on the head, and their thyroids taken according to the protocol in Table I.

TABLE I.

PROTOCOL FOR NAI^{131} INJECTION OF ANIMALS

Interval between NaI^{131} injection and thyroid dissection	Number of rats	NaI^{131} injected/animal microcuries
0.5	3	200
1	4	100
3	3	150
6	3	100
8	5	100
11	4	100
15	4	100
20	4	100
24	2	50
30	4	100
50	2	50
72	3	100

Pooled thyroid glands of each group were weighed and their radioactivity determined using a well-crystal scintillation counter that maintained a constant geometry and efficiency.

Each group of pooled thyroids was then put into a dialysis sac (Visking cellulose tubing 20/32) and dialysed 24 hr against 100 ml of precooled water at 0^{o}C. The introduction of glands into the sac was done through a small funnel in order to avoid any contact of glands with the upper part of the sac and therefore to eliminate possible contamination of the dialysate with traces of I^{131}-thyroglobulin remaining on the walls of the sac. This point is important in view of the small percentage of total I^{131} represented by the dialysate. The tubing was then double knotted at both ends in such a fashion as to eliminate most air bubbles and to leave between the double knots at each end a short length of tube that would provide a visual check as to whether the inner knots had leaked. The ends were then joined to give the final sac a ring form.

Pilot experiments with an apparatus that replaced with fresh precooled water the dialysate volume of 1 ml every 9 minutes and collected the effluent dialysate in fractions of 5 ml, showed the curve in Figure 1 as a function of time and volume

of effluent dialysate.

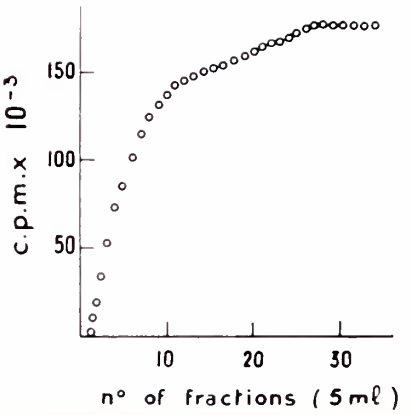

Figure 1. Continuous dialysis at a constant rate of 1 ml/9 minutes of I^{131} labeled rat thyroid glands (24 hours) against precooled water. Ordinate: cumulative radioactivity in the dialysate (counts/minute).

On this basis, it was assumed that at least 95% of the non-protein bound iodine had passed into the dialysate at 24 hours. Free T_4 and T_3 do not normally diffuse out in dialysis against water, these compounds being weakly soluble and strongly bound to thyroglobulin and other proteins. They dialyze out against human serum (20).

As the relative percentages of the iodocompounds dialyzed remained constant after the first 4-8 hours, it was assumed that the several per cent of free activity that remained in the glands after 24 hours did not constitute one sole compound but was distributed as in the dialysate.

After counting, the dialysates were concentrated under reduced pressure at a temperature lower than 40°C in a rotary evaporator until almost dry (volume about 0.5 ml). They were then transferred to a 1 cm^2 origin spot on whole sheets of Whatman No. 1 chromatographic paper (47 X 56 cm). Five micrograms each of MIT, DIT, T_3 and T_4 were added as carriers and descending chromatography in 1-butanol-acetic acid-water (78:5:17) was performed along the short dimension of the paper up to the opposite edge (17-18 hours at 18°C).

Developed chromatographs were air-dried at room temperature until no odor of acetic acid could be detected and trimmed to a width of 40 cm in the direction corresponding to chromatography.

Electrophoretic migration at right angles to the chromatographic direction was carried out in pyridine-acetic acid-water buffer (1:10:289, pH 3.65) according to Katz, Dreyer, and

Anfinsen (23) in an apparatus constructed in the laboratory according to the plans of these authors and supplied by the engineering staff of the National Institutes of Health, Bethesda, Maryland.

Dilutine 140[5] was used as a coolant and as a heat-transfer medium to the running water which circulated in stainless steel cooling coils. The considerable volume of the buffer (30 l) and the Dilutine (35 l) and convection currents in the coolant prevented problems due to overheating during electrophoresis.

Chromatographs, prepared as described above, were evenly wetted with the pyridine buffer by spraying with an atomizer. The origin and line of chromatographic migration were not directly wetted but allowed to imbibe from either wetted side, so as to concentrate the chromatographically separated products into a tighter band. If the origin would not wet in this manner, a drop of buffer was directly applied, for areas of the paper not wetted by the buffer will be wetted by the Dilutine coolant resulting in trailing and faulty migration. After 2 hours of electrophoresis at 2500 volts (average current for 40-cm wide Whatman No. 1 paper was 65-70 milliamperes), the paper was air-dried and then warmed in a 45°C oven to drive off traces of pyridine and acetic acid. It was then

autographed for 7-8 days on Kodak no-screen X-ray film (30 X 40 cm) with the paper sandwiched between two sheets of film and stapled. Position of the staples served to align paper and films for the procedure described below.

Areas of the chromatoelectrophoretograms corresponding to spots revealed on the films (Figure 4) were cut out and counted in a well-type scintillation spectrometer with automatic sample changing, permitting long enough counting times (60-120 minutes in certain cases) to achieve a statistical error of apporximately 1%.

Two areas of the paper taken in a region devoid of film-detectible radioactivity (blanks) were counted for the same time and their mean radioactivity was subtracted from that of areas corresponding to each radioactive spot.

The rest of the paper, i.e., that part not corresponding to any definite spot was then cut into convenient pieces and counted in the same manner to determine the total radioactivity on the paper. The percentage of inorganic iodide in the dialysate was determined by unidimensional electrophoresis and counting, after radio-autography, in the same fashion.

After dialysis, the thyroid tissue was ground with glass powder and hydrolyzed with pancreatin (Viobin 4X USP) added

in a weight ratio of 1:20 in 0.2 M NH_4HCO_3, pH 8.6, for 24 hours at 37°C. An aliquot of each hydrolysate with appropriate carriers (MIT, DIT, T_4, I^-, 5 micrograms each) was subjected to descending paper chromatography (Whatman No. 1) in the butanol-acetic acid solvent mentioned above. The distribution of radioactivity on the chromatograms was determined with an automatic scanning device equipped with a G-M counter. Areas under peaks were measured by planimetry and the percentage of radioactivity in each peak was calculated.

RESULTS

The percentage uptake for each time interval after I^{131} injection was divided by the average thyroid weight of each such group to obtain the percentage uptake per milligram of thyroid tissue. The results are shown in Figure 2.

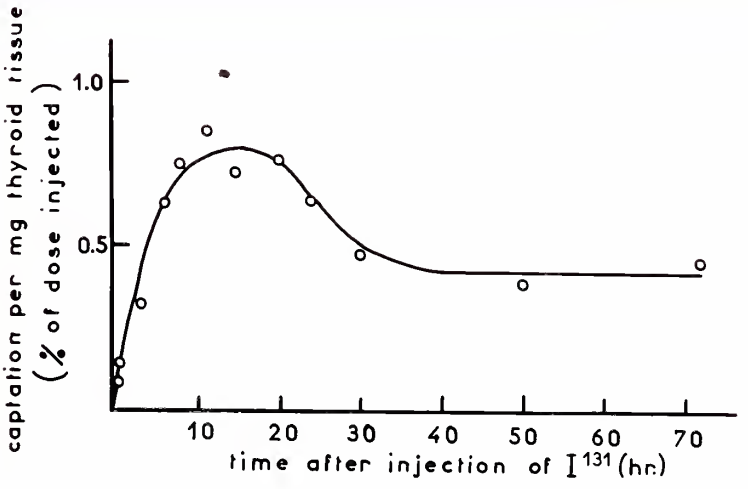

Figure 2. Uptake of I^{131} by the thyroid glands of rats injected with NaI^{131} from 30 minutes to 72 hours before death. Uptake (captation) is expressed as % injected dose/mg thyroid tissue.

It can be seen that the uptake is maximal between 10 and 20 hours. This maximum represents an uptake of about 8% for an average gland of weight 10 mg. This would indicate that the rats were receiving an adequate amount of iodine in the diet. Figure 3 shows what percentage of the total uptake was in the 'compartiment libre,' i.e., that which dialyzed at $0^{o}C$ and, to another scale, that percentage of this free activity which was organic.

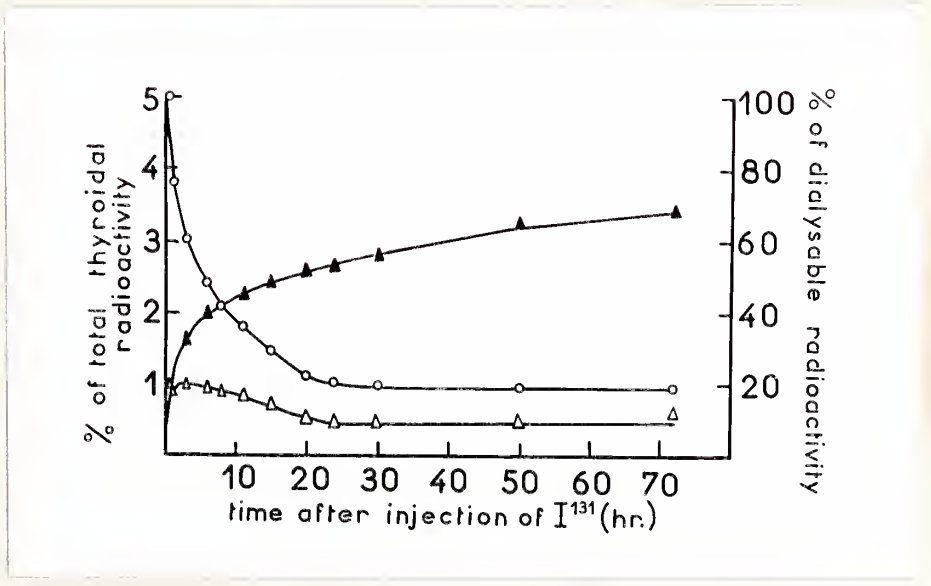

Figure 3. Distribution of radioactivity in the dialysates of rat thyroid glands from 30 minutes to 72 hours after the injection of I^{131}. Radioactivity of the total dialysable iodine (—O—) and of the free iodo-organic fraction (—△—) as percent of the total radioactivity of the glands. The curve (—▲—) shows dialysable iodo-organic activity as percent of total dialyzed (scale on right).

It is seen that the total activity of the 'compartiment libre' diminishes monotonically as that proportion which is organic increases. Radioautographs of chromato-electrophoretograms of the 'compartiment libre' of rat thyroid glands 1, 11, and 72 hours after I^{131} injection are shown in Figure 4. The diagram in lower right of Figure 4 is a schematic representation of the position after chromato-electrophoresis of the iodocompounds most frequently encountered as dialysable (or TCA-soluble) material in the thyroid. Besides MIT and DIT, lettering of these compounds was arbitrarily done from A to E beginning with those having the lower R_f in BA5. Compounds corresponding to spots labeled in lower case from a to e were not investigated in the present study.

Iodocompounds of group A do not migrate in the BA5 solvent system but are separated in electrophoresis. Those of group B have a chromatographic behavior roughly similar to that of MIT and DIT but different electrophoretic mobilities. The C group contains iodocompounds, the R_f of which is intermediate between 0 and that of inorganic iodide, and whose electrophoretic mobilities differ widely.

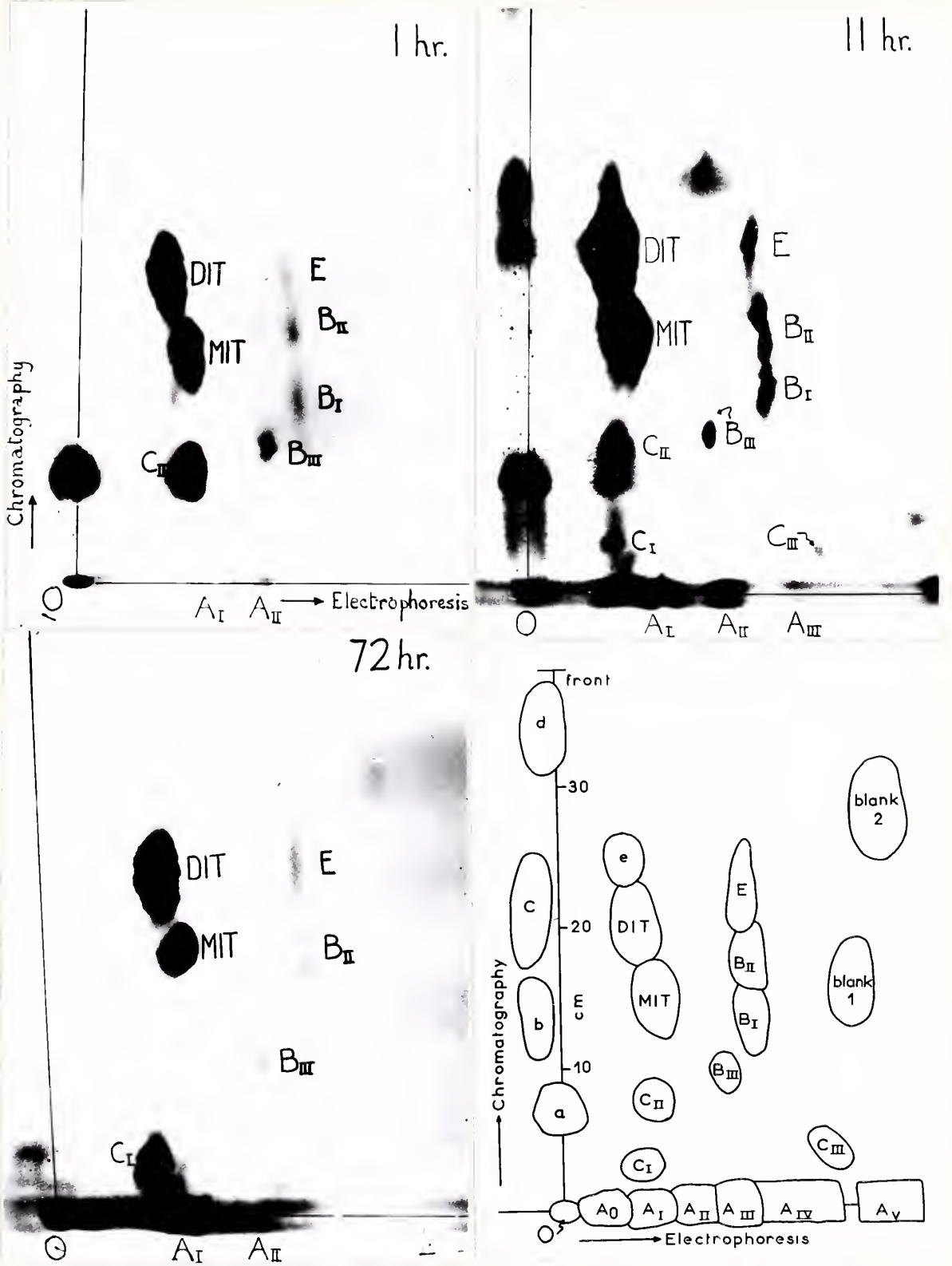

Figure 4. Autoradiographs of chromato-electrophoretographs of dialysates at 0° C. of rat thyroid glands 1, 11, and 72 hours after I^{131} injection. C is zone of deposition of the concentrated dialysate. Arrows indicate directions of migration in chromatography (BA5) and, for electrophoresis, the direction of the negative pole. The schematic drawing indicates the positions of the free iodocompounds most frequently encountered as dialysable material in rat thyroid gland. Compounds not labeled in the autoradiographs, or labeled lower case from a to e were not investigated in the present study. For explanation of other labeling, see text.

Peptides B_I and B_{II} gave MIT and DIT on pancreatic hydrolysis. As they were also present in ox thyroid glands, their isolation in a pure form was accomplished according to a method previously described (19). B_I (ox) showed the following amino acid composition: aspartic acid, glutamic acid, serine, glycine, alanine, valine, phenylalanine, the leucines, tyrosine, MIT, and sometimes DIT.

Studies done with C_I and C_{II}, labeled by I^{131} in vivo and purified by preparative chromato-electrophoresis on Whatman No. 3MM paper, indicated that C_{II} is hydrolysable by pancreatin with difficulty, giving only MIT as a labeled product (Figure 5).

C_I on hydrolysis yielded DIT and a compound migrating in the same position as C_{II} in BA5 (Figure 5) and t-amylol saturated with 2N NH_4OH ($R_f = 0$). As C_{II} does not migrate in alkaline organic systems but does migrate with iodide in butanol-acetic acid solvents, its detection is not usually accomplished with ordinary chromatographic systems: its radioactivity is attributed to inorganic iodide. The other spots (A, E) were presumed to be iodopeptides as they gave MIT or MIT + DIT on pancreatic digestion. Further characterization of these compounds was not undertaken as their very low concentration precluded

isolation of a sufficient quantity.

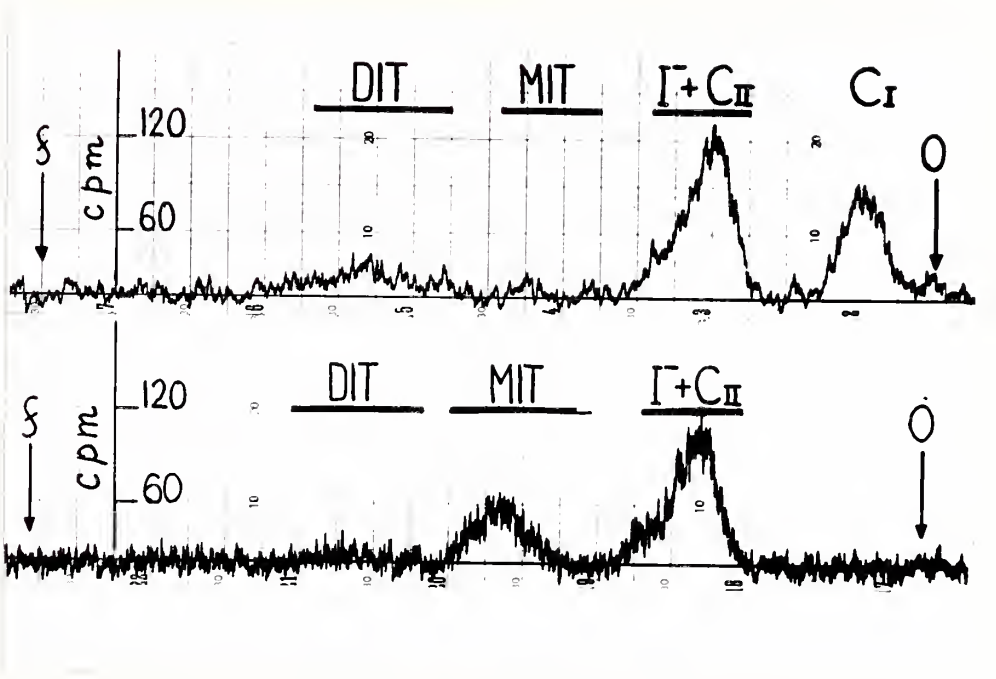

Figure 5. Scanning of the radioactivity of chromatograms (BA5) done with pancreatic digests of iodocompounds C_I (above) and C_{II} (below). The concentrated dialysate of thyroid glands of rats injected with NaI^{131} (100 uc) 14 hours prior to sacrifice was analyzed by chromato-electrophoresis and autoradiography. Areas corresponding to C_I and C_{II} were cut in small pieces and digested with pancreatin. Solid bars indicate position of carriers (I^-, MIT, DIT); 0= origin, f= solvent front. Ordinate: radioactivity in counts/minute. Abscissa: length of chromatographic migration, one division corresponds to 1 cm.

The relative intensities of the chromato-electrophoretic spots of the dialysable radioactivity differ according to the time elapsed after I^{131} injection (Figure 4). In order to quantify the concentration relationships between the various compounds, the following procedure similar to that proposed by Pitt-Rivers (16) for calculating estimated RSA was used.

The radioactivity represented by each spot, as a percentage of the total injected, was calculated by multiplying the fraction it represented of the radioactivity of the 'compartiment libre' by the fraction the 'compartiment libre' represented of the injected total. The assumption was made that at 50-72 hours after I^{131} injection the ratio of I^{131} to I^{127} in the labeled compounds had approached its equilibrium value. The assumption was supported by the fact that from 24 hours on, the proportion of radioactivity in each iodocompound is practically constant. It is evident, then, that at equilibrium the concentration of each iodocompound is proportional to its I^{131} content. If, now, due to the lack of precise knowledge of the concentration of the corresponding I^{127}-iodocompound, the specific activity of each iodocompound at equilibrium is set equal to one, the specific activity at any given time relative to this final value can be calculated.

Accordingly, the relative specific activities of each of the free iodocompounds was calculated as a function of time, by dividing by the equilibrium percentage the percentage of the total injected radioactivity represented at each time by each free iodo-organic compound. The relative specific activities of bound MIT and bound DIT were calculated in the same manner from the percentage of MIT and DIT found at each time interval after I^{131} injection in the dialyzed pooled thyroid glands after pancreatic hydrolysis. (See Appendix section for mathematical definitions and sample calculations.)

In Figure 6 curves comparing the relative specific activities of C_{II}, free MIT, and bound MIT are plotted. It is seen that iodide taken up by the thyroid is first incorporated into iodopeptide C_{II} and later into free and bound MIT.

Figures 7 and 8 compare the relative specific activities of MIT and DIT in the 'compartiment libre' with those of MIT and DIT in thyroglobulin. Note should be made of the differences in scale of the ordinates in these figures: the relative specific activities of the free iodotyrosines are much greater, at early time intervals after I^{131} injection, than those of MIT and DIT in thyroglobulin. It should also be noted that although the curves

could be reasonably retraced to present the maxima of free and bound MIT in such a position as to imply by the criteria mentioned

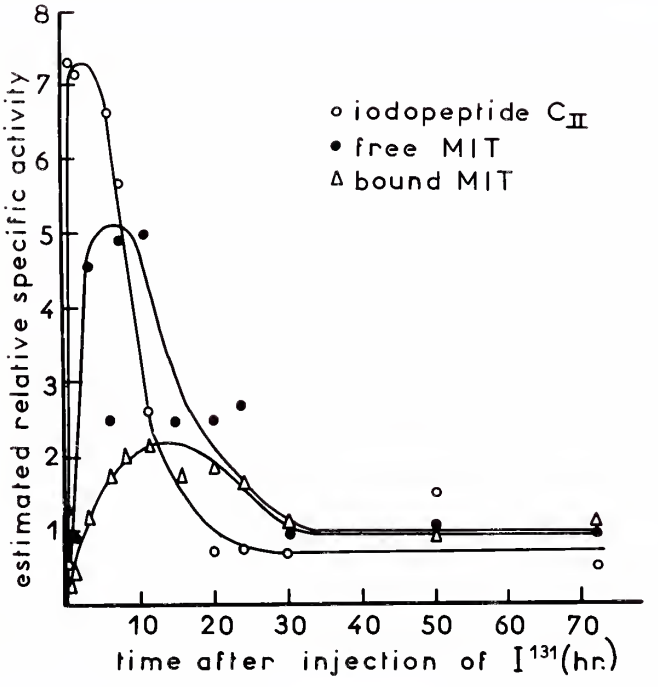

Figure 6. Estimated relative specific activities of iodopeptide C_{II}, free MIT (in the dialysates), and bound MIT (in the hydrolysates) of rat thyroid glands 30 minutes to 72 hours after injection of I^{131}.

earlier (17) that it could be a precursor of DIT, respectively free and bound, it would seem more logical to conclude that since I^{131} is incorporated maximally into DIT sooner than

would be possible if newly formed MIT were its only precursor, $DI^{131}T$ does not arise solely from the iodination of $MI^{131}T$.

Figure 9 presents the RSA curves of various iodopeptides. The group labeled A represents at least five iodopeptides (A_0, A_I,

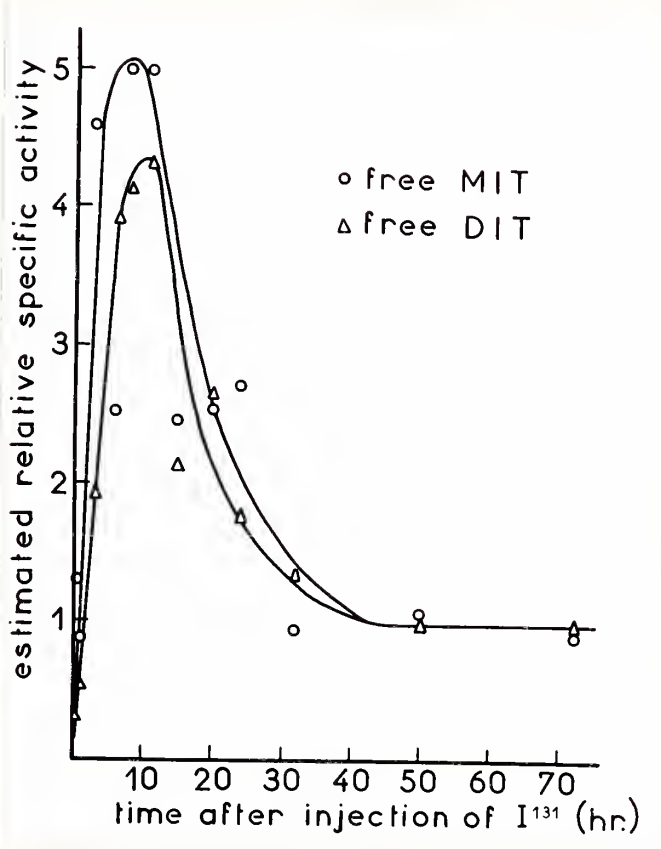

Figure 7. Estimated relative specific activities of free MIT and DIT in the dialysates of rat thyroid glands from 30 minutes to 72 hours after injection of I^{131}.

A_{II}, A_{III}, A_{IV}, A_V, Figure 4), the radioactivities of which were added to obtain their total RSA after it became evident that their

specific activities taken separately showed identical behavior.

Four components similarly compose the iodopeptides group B (B_I, B_{II}, B_{III}, E). Their RSA curve is seen to be

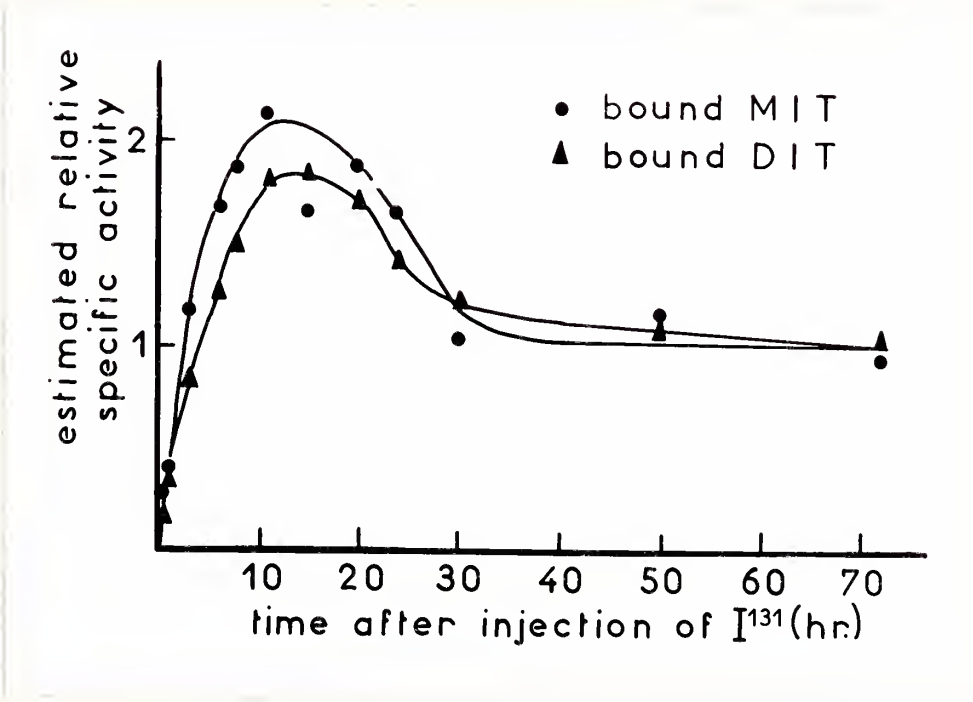

Figure 8. Estimated relative specific activities of bound MIT and DIT in the hydrolysates of rat thyroid glands from 30 minutes to 72 hours after injection of I^{131}.

very similar to that of bound DIT.

The RSA curve of iodopeptides $C_I + C_{III}$ shows a form similar to that of group A but reaches its maximum at an earlier time.

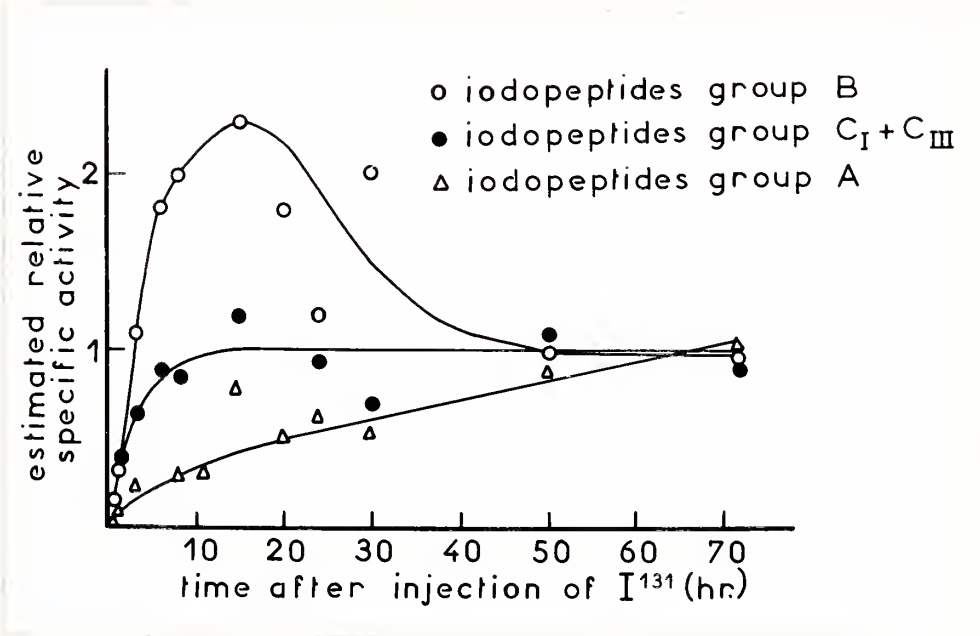

Figure 9. Estimated relative specific activities of iodopeptides groups B, $C_I + C_{III}$, and A (in the dialysates) of rat thyroid glands from 30 minutes to 72 hours after I^{131} injection.

One interesting sidelight is that C_I, and more often and in higher concentration C_{II}, are also noted in studies of thyroid slices incubated in vitro with NaI^{131}. Larger quantities of C_{II} are formed when the slices are placed in conditions under

which they are unable to organify iodine or to form DIT.

Although none of these iodopeptides has as yet been isolated in sufficient amount to permit more than token chemical investigation, suggestions as to their origin and composition can be drawn from their chromato-electrophoretic behavior and specific activity curves. The low migration of the iodopeptides of group A in BA5 ($R_f = 0$), their complete exclusion from dextran gel of high cross-linkage (Sephadex G-25) and the relative slowness with which they are labeled, suggest that they are rather large peptides (M. W. = 5,000 to 15,000) derived from thyroglobulin. The similarity in chromatographic and electrophoretic behavior and the remarkable similarity of the relative specific activity curves between iodopeptides B and bound DIT suggests that these iodopeptides are released from thyroglobulin not containing iodothyronines.

DISCUSSION

The finding that the radioactivity of the dialysable iodo compounds of the thyroid is highest at the shortest time intervals after I^{131} injection and then descends continuously seems to implicate free iodo compounds as primary intermediates in the biosynthesis of the thyroid hormones. Concerning their distribution in the 'compartiment libre,' it has been shown that the percentage radioactivity in the total free iodo-organic fraction increases in the same time and tends to plateau.

The concurrent study of the estimated relative specific activities of the various compounds constituting this free iodo-organic fraction has demonstrated the following points: (1) iodo-compound C_{II}, which appears to behave as an iodopeptide, reaches its maximum RSA (3-4 hours) before any other iodo-organic compound of the gland; (2) free MIT reaches its maximum RSA after C_{II} (7-8 hours) and nearly at the same time as free DIT (9-10 hours); (3) thyroglobulin-bound MIT attains its maximum RSA 5 to 6 hours after that of free MIT, confirming the recent findings of Pitt-Rivers and Cavalieri (18). The value of the maximum RSA is higher for C_{II} than for free MIT, which

is in turn higher than that of bound MIT. The RSA of the group of peptides B reaches its maximum value approximately at the same time as bound DIT, in contrast to the peptides A and $C_I + C_{III}$, the RSA of which increases up to 72 hours, suggesting that they derive from the proteolysis of thyroglobulin.

According to the classical scheme, serum inorganic iodide trapped by the thyroid cell ('first iodide pool') is very rapidly and sequentially organified in thyroglobulin into first MIT and then DIT. These residues are presumably the precursors of thyroglobulin-iodothyronines in a coupling reaction. Non-perchlorate-dischargeable iodide has been recently assumed to constitute a second iodide pool (22) coming from the deiodination of free iodotyrosines split off during proteolysis of thyroglobulin and with a lower turnover rate than that of iodide in the first pool.

Our results showing a high value for the relative specific activity of iodopeptide C_{II} and free MIT at early time intervals after I^{131} injection may be interpreted within the framework of this scheme if it is postulated that a thyroglobulin fraction exists with a high turnover rate, different from storage thyroglobulin in the colloid. Proteolysis of this fraction would give rise to C_{II} from which, in turn, free MIT and perhaps free DIT would be

produced; a possible function of C_{II} might be that in this form MIT is protected from intrathyroidal deiodinase.

However, another hypothesis would explain the experimental data: a precursor of C_{II} could be directly iodinated from the inorganic iodide trapped from the serum, giving rise to C_{II}. C_{II} and its product, free MIT could be direct precursors of thyroglobulin. Pitt-Rivers and Cavalieri (18) have already suggested that free tyrosine could be iodinated in the gland to explain the high RSA of free MIT as compared to bound MIT. The similarity between the RSA curves of free MIT and free DIT is more difficult to interpret (see RESULTS section), as the present data do not permit a choice between the following immediate precursors of free DIT: free MIT just formed, preformed free MIT, C_{II}, tyrosine, or a small tyrosine peptide.

Since these hypotheses on the intermediate metabolism of the thyroid hormones concern amino acids which are eventually incorporated into thyroidal proteins, an integration of these hypotheses with present-day theories of protein synthesis would be desirable. There has been radioautographic evidence (24) that iodination and protein synthesis do not take place in the same part of the thyroid acinus nor with the same time course. Other workers

(25, 26) using isolated thyroid cells have concluded that iodination probably takes place within the cell. The following discussion assumes that it is most likely that protein synthesis and iodination take place within the cell prior to the secretion of the final products into the follicular colloid.

Let us look first at the fundamental necessities of protein synthesis, amino acid activating enzymes and soluble ribose nucleic acids, insofar as they apply to thyroidal iodo-amino acids.

Herzfeld (27) using sheep thyroid pH 5 preparations found active pyrophosphate (P^{32}) exchange into ATP in the presence of tyrosine and MIT and other amino acids, but not with DIT. He was unable to isolate amino acyl-sRNA. Alexander (28) has also noted that L-tyrosine and MIT enhanced PP-ATP exchange and that this reaction was not shown or was inhibited by DIT, T_0, T_3 and T_4. He also found that tyrosyl hydroxamate formation was inhibited by excesses of MIT and DIT, whereas thyronines and iodothyronines were without effect. He isolated tyrosyl-sRNA from incubation mixtures of crude thyroid tissue supernates. Attempts (29) to demonstrate direct incorporation of iodothyronines by partially purified thyroidal systems consisting of calf thyroid amino acid activating enzymes and sRNA with both rat and dog

liver and calf thyroid ribosomes have been unsuccessful.

From these reports it seems likely that thyroid tissue possesses an amino acid activating enzyme for MIT and possibly for DIT and that it might be identical with that for tyrosine. The implication of this for thyroglobulin synthesis is that preformed free MIT and DIT could be incorporated as are the non-iodinated amino acids in the formation of thyroglobulin. MIT and DIT could also be incorporated into thyroglobulin by another route: activated tyrosine transferred to sRNA could be iodinated while in that combination and then incorporated as MIT and DIT into protein. These compounds, isolated in dialysates as MIT-sRNA and DIT-sRNA would tend to be unstable and decompose in acidic chromatographic solvents to yield 'free' MIT and DIT. The present RSA data are compatible with either mechanism, a choice between the two depending on the rate of amino acid transfer to protein relative to the rate of formation of the iodotyrosyl-sRNA. If the rate of transfer of amino acids by the iodotyrosyl-sRNA intermediate were relatively rapid the former mechanism would tend to be favored, while the latter would be more likely if that rate were shown to be relatively slower.

How, then, are T_3 and T_4 formed? Given that MIT and

DIT are coupled with DIT to form T_3 and T_4 what are the forms of the reacting species? Prior to the discovery (21) of free MIHPPA and DIHPPA in thyroid tissue the most likely hypothesis was that random apposition of iodotyrosine residues contained in thyroglobulin resulted in iodothyronine formation, leaving an 'extra' serine or similar residue to mark the site of the α-ring (outer ring) donor. However, no analysis of thyroglobulin amino acid composition has shown the expected (30) 2 extra residues per mole. The experiment of comparing amino acid analyses of thyroglobulin for serine or a similar residue when T_3 and T_4 formation have been suppressed by thiocarbamide administration or iodide deprivation with the amino acid composition when T_3 and T_4 formation are permitted has never been performed.

Another hypothesis combining old with newer evidence would be that the reacting intermediates are the thyroglobulin-bound iodotyrosines and their iodohydroxyphenylpyruvate derivatives. If, as the present data and those of Pitt-Rivers (16) would indicate, the maximum RSA values of bound DIT and T_4, and bound MIT and T_3, respectively, are nearly contemporaneous, this would preclude the thyroglobulin-bound iodotyrosines as precursors of iodothyronines. Indeed, it would appear to

imply that these two compounds share the same precursor(s), most likely the respective iodotyrosine-sRNA compounds, the MIT-sRNA and DIT-sRNA referred to above.

Diiodohydroxyphenylpyruvate has been shown to combine aerobically with DIT to form thyroxine (31, 32, 33). Peptides containing DIT can replace free DIT to yield thyroxine-containing peptides. These aerobic enzymatic and non-enzymatic condensations in vitro suggest that free DIHPPA and MIHPPA donate the α-ring to the iodotyrosyl-sRNA precursors of the thyroidal thyronines T_3 and T_4. DIHPPA and MIHPPA probably arise in thyroid tissue from free DIT and MIT through transamination with -ketoglutarate or perhaps through L-amino acid oxidase. the possibility also exists that 4-hydroxyphenylpyruvic acid (HPPA), formed from tyrosine by the reaction(s) above, is directly iodinated to MIHPPA and DIHPPA by iodide ion in a non-oxidative mechanism similar to the one in Figure 10.

That MIT-sRNA could also intervene in the biosynthetic process, to take the place of DIT-sRNA in combining with the iodohydroxyphenylpyruvates is suggested by the finding of small quantities of T_2 (5, 34, 35) and 3,3',5'-T_3 (34, 35) in hydrolysates of thyroid tissue.

A possible explanation for the low concentration of these compounds lies partially in the relatively lower concentrations of

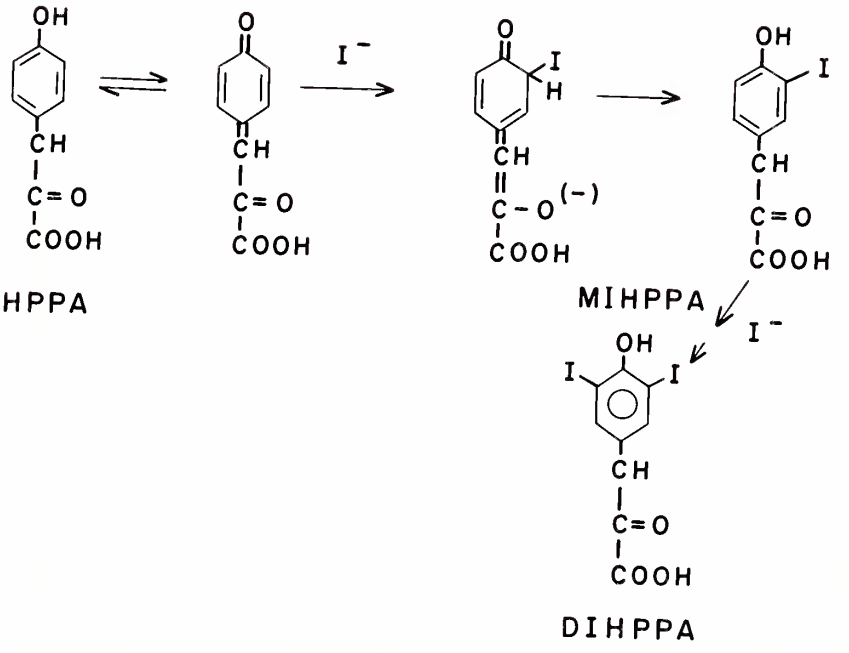

Figure 10. Possible mechanism for the formation of iodohydroxyphenylpyruvates showing iodination of 4-hydroxyphenylpyruvate by iodide ion. Unlabeled compounds are hypothetical reaction intermediates. (For other mechanisms see text.)

the free monoiodinated precursors. Probably more importantly, the absence of one of the ortho-halogens on the benzene ring in

MIT would make the 'phenoxide' ion less electronegative, resulting in decreased activation for nucleophilic substitution at the 1 position in the iodohydroxyphenylpyruvates and lowering the reaction rates for the formation of T_2 and T'_3. Figure 11

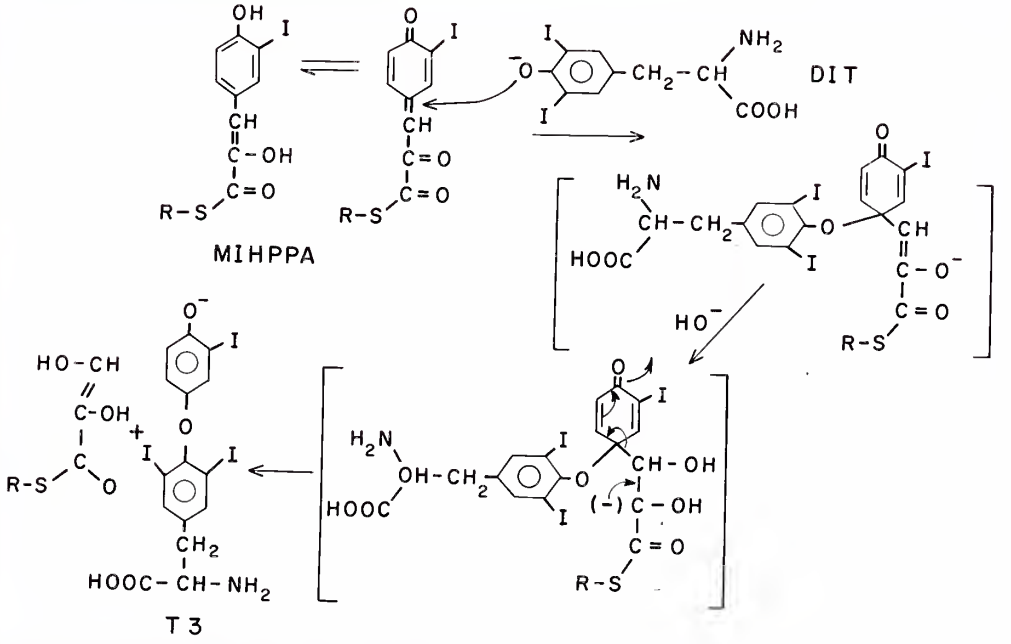

Figure 11. Possible mechanism for the general 'coupling' reaction leading to the formation of the iodothyronines. Shown is a condensation between 3,5-diiodotyrosine and 3-iodo-4-hydroxyphenylpyruvic acid forming 3,5,3'-triiodothyronine. A sulfur enzyme or co-enzyme (indicated R-S-) is included for reasons explained in the text.

shows a possible mechanism for the coupling reaction forming the diphenyl ether from the iodotyrosines and the iodohydroxyphenylpyruvates. Although unnecessary in vitro, a sulfur enzyme or co-enzyme is shown in the mechanism, as such an intermediate could facilitate the in vivo reaction by allowing distribution of negative charge onto the carboxyl oxygen.

In Figure 12 some of the thyroidal biosynthetic pathways proposed in this paper are pictorially combined. The iodination of either free tyrosine or tyrosyl-sRNA is suggested by the alternate pathways a, b or a', b', c', respectively. The iodinating species is shown as I^+; for the sake of clarity the mechanism elaborated in Figure 11, that the iodohydroxyphenylpyruvates, and thence the iodotyrosines, might be formed by iodination of hydroxyphenylpyruvic acid with iodide ion, is not included.

The coupling reaction between the iodotyrosyl-sRNA compounds and the iodohydroxyphenylpyruvates is indicated by the four dashed lines. Solid arrows indicate other reactions, with no implication as to reversibility of reaction. Thyroglobulin-bound amino-acids, shown in the right-hand column, have been isolated from mammalian thyroid tissue, as have the free iodohydroxyphenylpyruvates, free amino acids and tyrosyl-sRNA.

Presumptive evidence for the presence of MIT-sRNA and weaker presumptive evidence for DIT-sRNA has been presented.

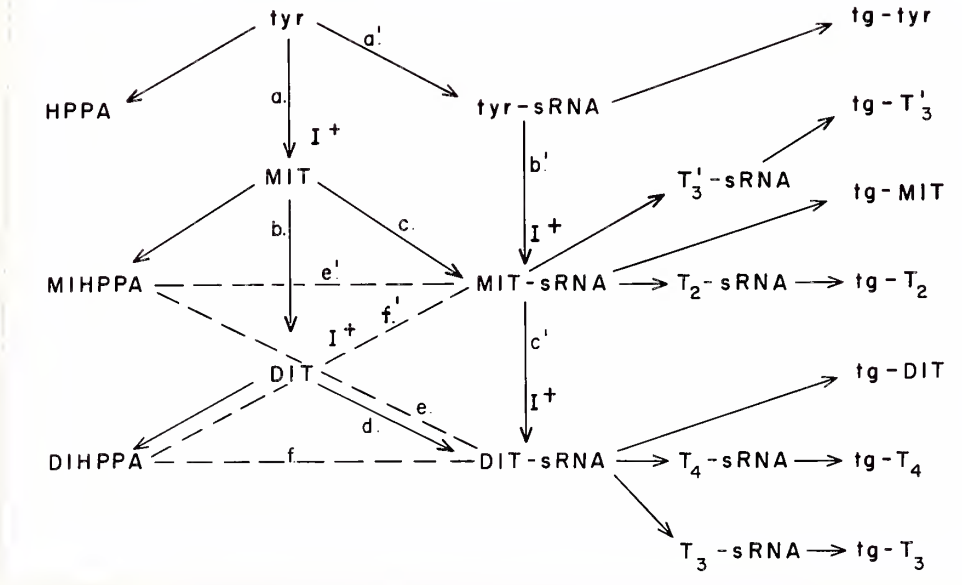

Figure 12. Possible pathways for the biosynthesis of the thyroid hormones. Abbreviations explained in text[2]; tg- means thyroglobulin bound. Condensation reactions suggested by dashed lines, all others by solid line. Letters a, b, c, d, and a', b', c' indicate two alternate pathways for organification of iodine and amino acid transfer to sRNA. An explanation for the relative preponderance of condensations e, f over e', f' is given in the text.

SUMMARY

The time course of appearance in rat thyroid glands after single injection of I^{131} as total thyroidal iodine, free iodide, free iodo-organic fractions and bound iodo-organic compounds has been investigated by the method of dialysis and chromato-electrophoresis (20). The relative specific activities of free MIT, free DIT, various free iodopeptides, bound MIT, and bound DIT have been determined. It has been shown that the most highly labeled iodocompound at early times after I^{131} injection is a consistently isolated free iodopeptide. Following in rapidity and extent of labeling are free MIT and free DIT; the RSA maxima for the thyroglobulin-bound iodotyrosines follow that of the free species by 5-6 hours.

The present data corroborate the experimental findings of Pitt-Rivers (16) and others (14) with respect to the activity-time relationships of I^{131} labeling of thyroglobulin-bound MIT and DIT; the findings of Bois and Larsson (15) that the $MI^{131}T$/$DI^{131}T$ ratio remains constant for one week after I^{131} injection are therefore not confirmed. Mechanisms (see Figures 11 and 12) are offered for the biosynthesis of thyroxine and triiodo-

thyronine in rat thyroid gland. On the basis of these hypotheses the findings of Pitt-Rivers (16) that RSA curves for T_3 and T_4 were similar in shape and maxima to those of thyroglobulin-bound MIT and DIT, respectively, find an explanation. Studies (10, 11) showing random labeling of I^{131} in both rings of T_3 and T_4 isolated from rabbit thyroid can be interpreted in the light of these hypotheses and the present experimental findings, although definite conclusions cannot be drawn until data regarding the rate of attainment of isotopic equilibrium in rabbit thyroid gland have been obtained and compared to those of the rat thyroid gland used here.

APPENDIX

SAMPLE CALCULATIONS

Let

$C_{(t)}$ = the percentage uptake of injected I^{131}/mg thyroid tissue,

$F_{(t)}$ = the percentage thyroidal radioactivity in the 'compartiment libre,'

$O_{(t)}$ = the percentage free radioactivity in the iodo-organic fraction of the 'compartiment libre,'

$r_{(t)}^{x}$, $r_{(t)}^{MIT}$, etc. = the percentage radioactivity in each component compound of the free iodo-organic fraction, and

$P_{(t)}^{x}$, $P_{(t)}^{MIT}$, etc. = the percentage of the total injected radioactivity represented by each free iodo-organic compound.

Then, for any given time interval after I^{131} injection, denoted by the subscript (t), one can relate the measured datum, $r_{(t)}^{x}$, to the total radioactivity injected by the formula

$$P_{(t)}^{x} = r_{(t)}^{x} \times C_{(t)} \times F_{(t)} \times O_{(t)}.$$

Since, at equilibrium, the percentage radioactivity of any free iodo-organic compound relative to the injected total becomes proportional to its representation in the stable, unlabeled form, $P_{(t)}^{x}$ divided by $P_{(t=\text{equilibrium})}^{x}$, (defined as

$$P_{(t=\text{equilibrium})}^{x} = P_{(eq)} = P_{(t=50)}^{x} + P_{(t=72)}^{x} \quad / 2$$

for reasons explained in the text) is proportional to the specific activity (SA) of compound x at any time (t):

$$SA_{(t)}^{x} = K \frac{P_{(t)}^{x}}{P_{(eq)}^{x}}, \text{ where K is constant;}$$

or,

$$\text{Relative SA} = \frac{P_{(t)}^{x}}{P_{(eq)}^{x}} .$$

To give a numerical example, with x = MIT, for the time interval 6 hours after injection of I^{131} (data from Table 2):

$$P_{(6)}^{MIT} = r_{(6)}^{MIT} \times C_{(6)} \times F_{(6)} \times O_{(6)}$$

$$P_{(6)}^{MIT} = 13.4 \times (0.65 \times 10^{-2})(2.4 \times 10^{-2})(39.0 \times 10^{-2})$$

$$= 8.1 \times 10^{-4}$$

$$P_{(eq)}^{MIT} = \frac{P_{(50)}^{MIT} + P_{(72)}^{MIT}}{2} = \frac{0.00034 + 0.00031}{2}$$

$$= 3.2 \times 10^{-4}$$

$$\text{Relative } SA_{(6)}^{MIT} = \frac{P_{(6)}^{MIT}}{P_{(eq)}^{MIT}} = \frac{8.1 \times 10^{-4}}{3.2 \times 10^{-4}} = 2.5$$

TABLE 2

$P_{(t)}^{x}$ AND RELATIVE SPECIFIC ACTIVITY CALCULATED FROM C, F, O, AND $r_{(t)}^{x}$. DATA FOR 6, 50, AND 72 HOURS AFTER NaI^{131} INJECTION.

Percentage radioactivity				Iodo compound					
								Iodo peptides group	
t (hrs)	F	C	O	MIT	DIT	$C_I + C_{III}$	C_{II}	A	B
6	2.4	0.65	39.0	13.4[a] 0.00081[b] 2.51[c]	56.0 0.00034 3.91	13.9 0.00024 0.88	3.5 0.00021 6.6	7.9 0.00048 0.85	2.0 0.00012 1.8
50	0.96	0.45	65.0	12.2 0.00034 1.06	32.1 0.0009 1.04	8.9 0.00025 0.92	1.7 0.00005 1.49	17.7 0.00049 0.88	3.1 0.00009 1.3
72	0.96	0.42	69.0	11.0 0.00031 0.94	30.3 0.00084 0.97	10.7 0.00030 1.11	0.6 0.000016 0.50	23.1 0.00064 1.12	1.5 0.000043 0.66
$P_{(eq)^x}$				0.00032	0.00087	0.00027	0.000032	0.00056	0.000065

(a) $r_{(t)}^{x}$ Value. (b) $P_{(t)}^{x}$ Value. (c) Relative $SA_{(t)}^{x}$.

Note: Abbreviations C, F, O, $r_{(t)}^{x}$, $P_{(t)}^{x}$, and $SA_{(t)}^{x}$ as explained in text of Appendix.

FOOTNOTES

FOOTNOTES

1

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2

Abbreviations: MIT, 3-iodotyrosine; DIT, 3,5-diiodotyrosine; T_2, 3,3'-diiodothyronine; T_3, 3,5,3'-triiodothyronine; T_3', 3,3',5'-triiodothyronine; T_4, thyroxine; MIHPPA, 3-iodo-4-hydroxyphenylpyruvic acid; DIHPPA, 3,5-diiodo-4-hydroxyphenylpyruvic acid; ATP, adenosine triphosphate; sRNA, soluble ribosenucleic acid; BA5, 1-butanol--acetic acid--water (78:5:17); RSA, relative specific activity.

3

Throughout this paper the adjective 'bound' will imply that the compound under consideration is contained in a peptide sequence and liberated only by enzymatic digestion.

4

That portion of this paper dealing with the experimental

investigation has been published (36) as a communication from the laboratory noted above.

5 Dilutine 140 is the trade name of Shell-Berre for a light petroleum ether fraction (flash point 70°C).

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